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RESEARCH NOTE

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Expression of α-Galactosidase Gene from *Leuconostoc mesenteroides* SY1 in *Lactobacillus brevis* 2.14

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Abstract α-Galactosidase gene (*aga*) from *Leuconostoc mesenteroides* SY1 was expressed in a heterologous host, *Lactobacillus brevis* 2.14 using an *Escherichia coli-Leuconostoc* shuttle vector, pSJE. pSJEaga (pSJE carrying *aga*) was introduced into *Lactobacillus brevis* 2.14 by electroporation and transformation efficiency was 1.1×10^3 per μg DNA. *L. brevis* transformants (TFs) showed higher α-galactosidase (α-Gal) activities than cells containing pSJE. Transcription levels of *aga* in *L. brevis* 2.14 grown on different carbon sources (1%, w/v) were examined by slot blot analysis. *Aga* transcript levels and α-Gal activities were higher in cells grown on melibiose, raffinose, and galactose than cells on glucose, sucrose, and fructose. Western blot result showed that *L. brevis* 2.14 harboring pSJEaga produced much more α-Gal when grown on melibiose than on glucose.

Keywords: *Leuconostoc* α-galactosidase, heterologous gene expression, *Lactobacillus brevis*, electroporation, pSJE

Introduction

Lactobacillus brevis, a heterolactic fermenter, is an organism frequently isolated from various fermented vegetables including *kimchi* and sauerkraut (1,2). It produces carbon dioxide and ethanol in addition to lactic acid from carbon sources such as glucose, thus contributing to the unique taste of fermented foods (3). *L. brevis* is one of the major lactic acid bacteria (LAB) responsible for *kimchi* fermentation, and isolated from many different types of *kimchi* (4,5). *aga* encodes α-Gal and α-Gal (α-D-galactoside-galactohydrolase, EC.3.2.1.22) catalyzes the hydrolysis of 1,6-linked α-galactose residues from oligosaccharides and polymeric galactomannans (6). α-Gal has some important industrial applications. In the sugar beet industry, α-Gals have been used to increase the sucrose yield by eliminating raffinose, which prevents the crystallization of beet sugar (7). α-Gals also have been employed to reduce raffinose and stachyose contents in soybeans. Both sugars are known to cause flatulence, one of the major drawbacks of soy products, which must be overcome if more wide consumption of soy products is realized (8). We previously constructed an *Escherichia coli-Leuconostoc* shuttle vector, pSJE (9), and tried *aga* expression in *Leuconostoc citreum* (10). In this communication, we report heterologous expression of *aga* from *L. mesenteroides* SY1 in *L. brevis* 2.14.

Materials and Methods

Bacterial strains and culture conditions *L. brevis* 2.14 (11) was grown in MRS (Difco, Becton Dickinson Co., Sparks, MD, USA) broth containing different carbon sources (1%, w/v) or on MRS agar plate (1.5%, w/v) at 37°C. *E. coli* DH5α was grown in Luria-Bertani (LB) broth at 37°C with vigorous agitation. Erythromycin (Em) was added at 200 μg/mL for *E. coli* and 5 μg/mL for *L. brevis*, respectively.

DNA isolation and manipulation Plasmid DNA from *E. coli* was prepared using QIAprep spin miniprep kit (Qiagen, Valencia, CA, USA), and plasmid DNA from *L. brevis* was isolated by the method of O'Sullivan and Klaenhammer (12).

Electroporation Introduction of plasmid into *E. coli* and *Lactobacillus* was done by electroporation method using Gene Pulser II (Bio-Rad, Hercules, CA, USA). Frozen competent *L. brevis* 2.14 cells were prepared as described previously (13). Frozen *E. coli* competent cell preparation and electroporation procedures were followed by the method of Dower *et al.* (14).

Slot blot analysis Total RNA was isolated from *L. brevis* cells grown on MRS media containing different carbon sources (1%, w/v). Cells were recovered when OD_{600} reached 0.8 and RNA was prepared from cells as described previously (10). A probe (952 bp) corresponding to the internal part of *aga* was obtained by polymerase chain reaction (PCR) as described previously (10).

α-Gal activity assay Cell extracts from *L. brevis* 2.14 were obtained as described previously (10). Protein concentrations were determined by the Bradford method (15) using a kit (500-0201EDU; Bio-Rad). α-Gal activity was measured by the method of Church *et al.* (16) using McIlvaine buffer. The method is based on the measurement of the absorbance at 400 nm caused by *p*-nitrophenol (PNP), which is released by the action of the enzyme from its specific substrate, *p*-nitrophenyl-α-galactopyranoside (PNPG).

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One unit of enzyme activity (U) was defined as the amount of enzyme that released 1.0 nmol of PNP from substrate PNPG per min.

Western blot Production of His-tagged α-Gal in *E. coli* BL21(DE3) and chicken anti-α-Gal IgY antibodies were described previously (10). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (17), using a 10% gel and 5 μg of each protein sample was loaded per lane. Transfer of separated proteins onto a Hybond-P PVDF membrane (Amersham Bioscience, Piscataway, NJ, USA) and following hybridization were done as described previously (10).

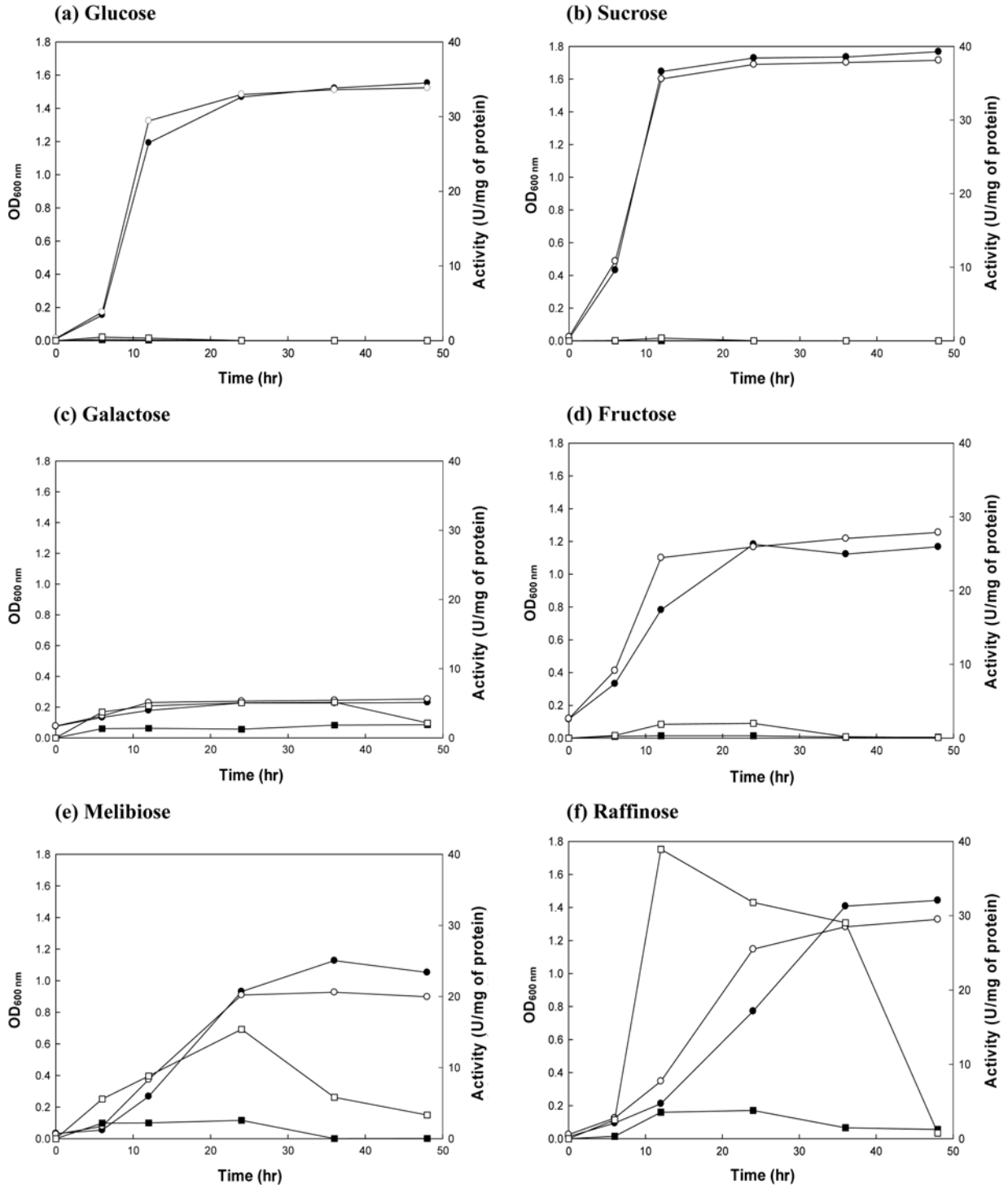

Fig. 1. Growth and α-Gal activities of *L. brevis* 2.14 TFs. *L. brevis* 2.14 was grown on MRS media containing different carbon sources (1%, w/v). Glucose (a), sucrose (b), galactose (c), fructose (d), melibiose (e), and raffinose (f). ● Growth of *L. brevis* 2.14 [pSJE]; ○ growth of *L. brevis* 2.14 [pSJEaga]; ■ α-Gal activity of *L. brevis* 2.14 [pSJE]; □ α-Gal activity of *L. brevis* 2.14 [pSJEaga].

Results and Discussion

pSJEaga, a derivative of pSJE containing *aga* gene in 2.5 kb fragment, was introduced into *L. brevis* 2.14 by electroporation. Transformants (TFs) were visible after 36 hr of incubation at 37°C on MRS agar containing Em (5 μg/mL) and transformation efficiency was 10^3 TFs/μg DNA, significantly lower than that of *E. coli* cells. The poor transformation efficiency is one of the major obstacles preventing strain improvements for LAB via genetic engineering. When plasmid preparation from a *L. brevis* TF was digested by *Kpn*I, a 2.5 kb fragment encompassing *aga* was generated, confirming the presence of pSJEaga in *L. brevis* 2.14 TFs (results not shown). *L. brevis* TFs grew rapidly on glucose, fructose, and sucrose, and reached stationary phase quickly but grew slowly on media containing galactose, melibiose, or raffinose (Fig. 1). Cells grown on raffinose had the highest α-Gal activity (38.9 U/mg protein) at 12 hr of incubation, then the activity gradually decreased, reaching 0.8 U/mg protein at 48 hr. Interestingly, cells grown on different sugars reached the highest enzyme activity at different time after incubation started: melibiose (15.4 U/mg protein) and fructose (2.0 U/mg protein) at 24 hr, galactose at 36 hr (5.2 U/mg protein), sucrose at 12 hr (0.4 U/mg protein), and glucose at 6 hr (0.5 U/mg protein). The maximum α-Gal activity (38.9 U/mg protein) observed in *L. brevis* 2.14 was much lower than that observed in *L. citreum* harboring the same plasmid (1,150 U/mg protein), corresponding to only 3% of the latter (10). *L. brevis* 2.14 [pSJEaga] cells grew poorly on melibiose, galactose, and raffinose, reaching the maximum absorbance of ca. 1.0 around 48 hr of incubation. Cells grown on glucose or sucrose reached more than 1.4 within 12 hr of incubation. Same results were observed in *L. citreum*. The poor growth of *L. brevis* 2.14 on galactose was probably caused by either problem in galactose uptake from the culture medium or lack of catabolizing pathways in the cell. Further studies are necessary to pinpoint the exact cause of poor growth. It has been reported that in *L. brevis* ATCC 367, galactose enters the cell by active transport energized by the proton motive force (18). Melibiose and raffinose seem to be easily transported into *L. brevis* cells and catabolized inside the cell. α-Gal produced by pSJEaga did not apparently contribute to the growth of *L. brevis* 2.14. *aga* mRNA levels in *L. brevis* 2.14 TFs grown on different carbon sources were determined by slot blot. Much more *aga* transcripts were synthesized in cells harboring pSJEaga whereas *aga* transcript was not detected in cells harboring pSJE (Fig. 2). Strong signals were observed from RNA samples from cells grown on melibiose, raffinose, and galactose, and weak signals from those from cells grown on glucose, sucrose, and fructose. The difference in the amount of RNA synthesized matched well with the measured specific α-Gal activities of recombinant cells on different sugars. The results also indicated that *aga* transcription in *L. brevis* was affected by the type of carbon source and this is an evidence for the operation of carbon catabolite repression (CCR) in *L. brevis* 2.14 as shown in *L. mesenteroides* (19).

Cells were recovered by centrifugation and protein extracts were prepared when culture reached at a growth stage where

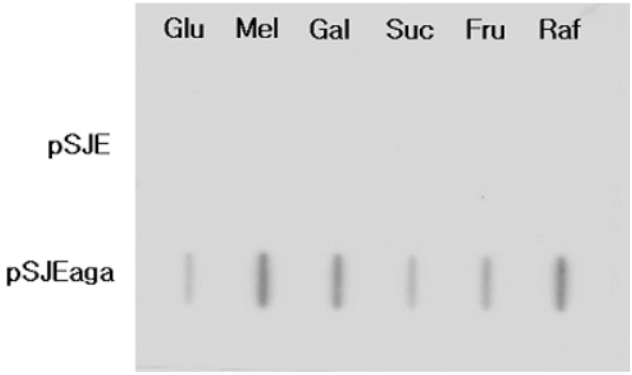

Fig. 2. Slot blot hybridization. *L. brevis* 2.14 was grown on MRS media containing different carbon sources (1%, w/v). RNA was prepared from cells harboring either pSJE or pSJEaga and 10 μg of each RNA was applied onto a nylon membrane using a slot blot apparatus. Glu, glucose; Mel, melibiose; Gal, galactose; Suc, sucrose; Fru, fructose; Raf, raffinose.

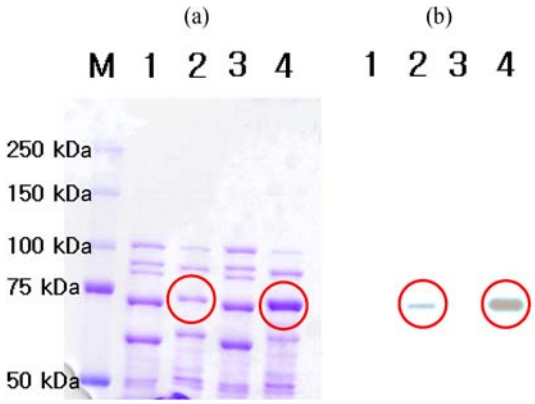

Fig. 3. SDS-PAGE and Western blot. *L. brevis* 2.14 was grown on MRS media containing either glucose or melibiose (1%, w/v) for 12 hr (glucose) or 24 hr (melibiose). Protein samples were prepared by sonication of cells and analyzed by SDS-PAGE (a) and Western blot (b) using 10% acrylamide gels. 1, *L. brevis* 2.14 [pSJE] grown on glucose; 2, *L. brevis* 2.14 [pSJEaga] grown on glucose; 3, *L. brevis* 2.14 [pSJE] grown on melibiose; 4, *L. brevis* 2.14 [pSJEaga] grown on melibiose.

α-Gal activity was high. Western blot results showed that *L. brevis* 2.14 [pSJEaga] produced a protein of ca 72 kDa (Fig. 3), which was not observed from *L. brevis* [pSJE]. The apparent molecular weight determined by SDS-PAGE was around 72 kDa, smaller than the expected size of α-Gal calculated from its sequence (84.3 kDa) (19). But the band must be α-Gal since it was the only protein detected by α-Gal-specific antibodies. Furthermore, the band intensity increased when cells grown on melibiose rather than glucose (Fig. 3b). The location of α-Gal on a polyacrylamide gel was circled in Fig. 3. α-Gal migrated to this location with other proteins of similar sizes. Unless aided by a special expression vector and further concentration (such as ammonium sulfate precipitation), it is usually difficult to observe a protein band derived from an introduced gene by SDS-PAGE because of generally low gene expression level in LAB. By alignment of coomassie blue stained gel and Western blot membrane, the location of α-Gal was guessed and circled.

All the results described so far clearly showed that *aga* was expressed and responsible for the increased α-Gal activities in *L. brevis* 2.14 TFs. More α-Gal was produced in cells carrying pSJEaga resulting in higher α-Gal activities than cells carrying pSJE judged from enzyme assays and Western blot results. Although the increased α-Gal production did not improve the growth of cells on media containing α-galactosides and the enzyme activity varied significantly depending on hosts, the recombinant strains might be useful to reduce raffinose and stachyose contents in soymilk, leading to production of fermented soy products with better digestability. In this respect, continued studies on the construction of recombinant strains are necessary. Especially, development of food-grade expression systems for LAB is highly desirable (20).

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